



Protocol

IncuCyte® Cell Cycle Assay

For quantification of cell cycle progression

This protocol provides an overview of the IncuCyte Cell Cycle Assay methodology which uses a genetically encoded ubiquitination-based lentiviral indicator (FUCCI). This assay is compatible with the IncuCyte®

Live-Cell Analysis System configured with a Red/Green Optical Module using your choice of cells and treatments.

Required materials

- IncuCyte® Cell Cycle Red/Green Lentivirus (Sartorius Cat. No. 4779)
- Flat bottom 96-well tissue culture plate (e.g. Corning Cat. No. 3595)
- Complete cell culture media for cell line of choice

Optional

- IncuCyte® Cell-by-Cell Analysis software module (Sartorius Cat. No. 9600-0031)
- Polybrene® (Sigma H9268)
- Poly-L-Ornithine (Sigma P4957) – optional, for non-adherent cell types

Transduction Protocol

Protocol for Immortalized Cell Lines

If you plan to use the IncuCyte® Cell Cycle Red/Green Lentivirus Reagent to generate stably-expressing clones or populations, please perform the "Optimizing Antibiotic Selection" step first. Optimizing MOI and transduction conditions are less important as the selection process will eliminate non- or low-expressing cells within the population.

1. Seed cells in growth media of choice at a density such that they are 15–35% confluent at time of infection. Incubate for 24 hours or until cells have attached to the plating surface.
2. Add IncuCyte® Cell Cycle Red/Green Lentivirus Reagent at desired multiplicity of infection (MOI = TU/cell) diluted in media ± Polybrene®. An MOI of 3 and Polybrene® concentration of 8 µg/mL is recommended for most cell types.
3. Incubate at 37° C, 5% CO₂ for 24 hours.
4. After incubation remove media and replace with fresh growth media. Return to incubator for an additional 24–48 hours, monitoring expression using an IncuCyte® Live-Cell Analysis System.
5. Harvest cells and expand, freeze, or seed at desired density for subsequent experiments. For stable selection, proceed to step 6.
6. (Optional) Remove media and replace with fresh growth media containing appropriate concentration of Puromycin determined from the kill curve (see section below, "Optimizing Antibiotic Selection").
7. Incubate for 72–96 hours, replacing media every 48 hours.
8. Maintain stable population in a maintenance concentration of selection media (e.g., complete media containing 0.5 µg/mL Puromycin).

Optimization Protocols Antibiotic Selection (optional)

In order to generate a stable cell line expressing the Cell Cycle Red/Green Reagent, determine the minimum concentration of antibiotic required to efficiently eliminate non-transduced cells using a range of Puromycin (typical antibiotic working concentration range for mammalian cells is 0.5–10 µg/ml, and effectiveness can be reached for most cell lines in 2–7 days).

Polybrene® Concentration

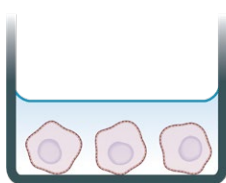
The cationic polymer, Polybrene®, may be used to increase the efficiency of transduction of certain cell types. Optimal Polybrene® concentrations will vary depending on the cell type used. Please note, Polybrene® can be toxic to certain cell types (e.g. primary neurons). The IncuCyte® Cytotoxicity Assay can be used to evaluate the toxic effect of Polybrene® on your cells.

Protocol for Primary Cells and Transient Assays

If you plan to use the IncuCyte® Cell Cycle Red/Green Lentivirus Reagent for transient assays, we recommend optimizing MOI and Polybrene® concentration for each cell type used (see "Optimization Protocols" section above). Once these steps are complete, follow the "Suggested Infection Protocol for Immortalized Cell Lines", steps 1 through 5.

Quick guide

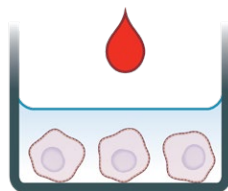
1. Seed cells



Cell Seeding

Seed cells in growth media and leave to adhere (4–24 hours). Cells should be 15–35% confluent at the time of transduction.

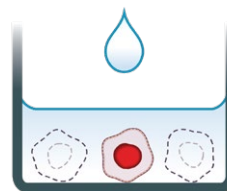
2. Transduce



Add IncuCyte® Cell Cycle Lentivirus Reagent

Add IncuCyte Cell Cycle Lentivirus Reagent diluted in media ± Polybrene®. After 24 hours, replace the media with fresh growth media. Monitor expression using the IncuCyte® Live-Cell Analysis System.

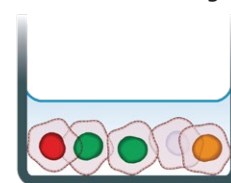
3. Apply selection



Generate a Stable Population or Clone

Apply Puromycin selection to derive a stable cell population or clone that expresses the genetically encoded cell cycle indicator.

4. Live-cell fluorescent imaging



Automated Imaging and Quantitative Analysis

Capture images (4x, 10x or 20x) in the presence of treatments in an IncuCyte® Live-Cell Analysis System configured with a green/red optical module. Analyze using integrated software.

Cell Cycle Assay Protocol

General guidelines

- Following cell seeding, place plates at ambient temperature to ensure homogenous cell settling.
- Remove bubbles from all wells by gently squeezing a wash bottle containing 70–100% ethanol with the inner straw removed, to blow vapor over the surface of each well.
- After placing the plate in the IncuCyte® Live-Cell Analysis System, allow the plate to warm to 37° C for 30 minutes prior to scanning.

Adherent Cell Line Protocol

1. Seed IncuCyte® Cell Cycle expressing cells (100 µL per well) at an appropriate density into a 96-well plate to achieve 20% confluency at time of assay. The seeding density will need to be optimized for the cell line used; however, we have found that 2,000 to 5,000 cells per well (20,000–50,000 cells/mL seeding stock) are reasonable starting points.
2. Allow cells to adhere to for 4–20 hours at 37° C. During this time the plate can be monitored in the IncuCyte® Live-Cell Analysis System to evaluate cell health and baseline assessment of cell cycle progression.
3. Optional: If interested in modulators of cell cycle, prepare treatments at 3x desired final assay concentration in complete growth media and add 50 µL/well.
4. Return plate to the IncuCyte® Live-Cell Analysis System for continued imaging and analysis.

Non-adherent Cell Line Protocol

1. Coat a flat bottom 96-well plate with relevant coating matrix. We recommend coating with 50 µL of 0.01% Poly-L-Ornithine solution. Coat plates for 1 hour at ambient temperature, remove solution from wells, and then allow plates to dry for 30–60 minutes prior to cell addition.
2. Count cells and prepare a cell seeding stock at the appropriate density. Seed your choice of cells (100 µL per well) at an appropriate density into the coated 96-well plate. The seeding density will need to be optimized for the cell line used but we recommend 10,000–30,000 cells per well (100,000–300,000 cells/mL). NOTE: If adding modulators of cell cycle, we recommend preparing treatments and adding these to the well prior to cell seeding in order to avoid heterogeneous cell seeding.

3. Optional: If interested in modulators of cell cycle, prepare treatments at 3 x desired final assay concentration in complete growth media and add 50 μ L/well.
4. Allow cells to settle at ambient temperature for 30 minutes or centrifuge at 50 x g for 1 minute, then place in IncuCyte[®] Live-Cell Analysis System for imaging and analysis.

Live-cell Imaging and Analysis

1. Using IncuCyte[®] software, schedule scans at desired frequency.
NOTE: Scan interval will vary depending on experimental paradigm (for cell cycle monitoring, this may require more frequent scans).
 - a. Scan type: Standard
 - b. Scan settings: select none for basic image capture. If using an IncuCyte[®] S3 licensed with the IncuCyte[®] Cell-by-Cell Analysis Software Module, select either the Adherent or Non-Adherent option within the scan settings for greater ease of data analysis.
NOTE: if using an IncuCyte ZOOM instrument select standard scan type.

- c. Channel selection: Phase, Red, and Green.
- d. Objective:
 - i. If using basic image capture we recommend either 10 x or 20 x objective.
 - ii. If Cell-by-Cell Analysis for non-adherent cells is enabled, only 20 x imaging is available.
 - iii. If Cell-by-Cell Analysis for adherent cells is enabled, only 10 x imaging is available.

Data Analysis

Analysis using Cell-by-Cell Analysis Software

1. If using adherent cells, use the Adherent Cell-by-Cell analysis type to create a new Analysis Definition

- a. Select representative images from both treated and control wells. Ensure majority of images have \geq 50% cell coverage to enable accurate masking.
- b. Begin by optimizing the Cell Boundary (with fluorescent channels turned off) by determining the optimal Seed mask (goal is to have one seed per cell) using the slider controls.
- c. Evaluate the Cell-by-Cell Mask and refine the parameters accordingly. Once you are happy with the parameters, click Preview All to ensure that the analysis parameters are appropriate for other time points or treatments selected.
- d. Once you have previewed all of the representative images and are satisfied with the parameters, complete the Launch wizard analysis to select the Scan Times and wells to be analyzed, as well as assigning an analysis definition name.
NOTE: If your experiment is in progress you will have an option to check "Analyze Future Scans" to perform real-time analysis.

- e. For further in-depth guidelines to aid with analysis, please see Analysis guideline document within user interface.

2. If using non-adherent cells, use the non-adherent Cell-by-Cell analysis type to create a new Analysis Definition

- a. Select representative images from both treated and control wells.
- b. Begin by setting an object diameter as measured with the measuring tool.
- c. Use the sliders for Threshold, Texture and Edge sensitivity to optimize the Cell-by-Cell mask to result in accurately masked cells.
- d. Once you have previewed all of the representative images and are satisfied with the parameters, complete the Launch wizard analysis to select the Scan Times and wells to be analyzed, as well as assigning an analysis definition name.
NOTE: If your experiment is in progress you will have an option to check "Analyze Future Scans" to perform real-time analysis.
- e. For further in depth guidelines to aid with analysis please see Analysis guideline document within user interface.

3. Data Interpretation (Cell-by-Cell Analysis)

- a. Once cell segmentation has been completed the data can be classified within the software based on red and green fluorescence intensity using a 2D scatter plot. Classification gates can be set to determine high and low green and red populations.
 - i. High red, low green—cells in G1 phase
 - ii. Low red, high green—cells in S/G2/M phase
 - iii. Low red, low green—M/G1 phase, and non-transfected
 - iv. High red, high green—G1/S phase
- b. After classification any identified sub-population can be viewed either as count or percentage of total population.

Analysis using Basic Analyzer

1. If Cell-by-Cell software is not available, process data using Basic Analyzer

- a. Select Phase/Green/Red/Overlap Image channels. Overlap will mask the yellow (G1/S) transition of the cell cycle.
- b. Select representative images from both treated and control wells.
- c. Begin by optimizing the phase segmentation with fluorescent channels turned off and clicking Preview Current. Adjust segmentation, cleanup and filter parameters as required.
- d. Subsequently optimize analysis masks for the Red and Green Image Channels, followed by Overlap. NOTE: Check overlap mask to be sure it is not over-estimating the number of "yellow" cells (you may need to filter or alter the both the Red and Green channel edge split).
- e. Once you are happy with the selected parameters for all channels, click Preview All to ensure these are appropriate for other time points or treatments selected.
- f. Once you have previewed all of the representative images and are satisfied with the parameters, complete the Launch wizard analysis by selecting the Scan Times, wells to be analyzed and an analysis definition name. NOTE: If your experiment is in progress you will have an option to check "Analyze Future Scans" (S3) or "Open-Ended" (ZOOM) to perform real-time analysis.

2. Data Interpretation (Basic Analyzer)

- a. After the analysis job is complete, the Red/Green/Overlap count (per image) may be plotted. Note, the Red and Green counts do not represent the pure populations because the cells masked as Overlap (Yellow) will be included in these counts.
- b. In order to normalize count data, it is easiest to plot Object Count (per image) and export raw data into a data analysis program (e.g. Excel).
 - i. To calculate the pure population of Red and Green cells, subtract the Overlap count from each count (e.g. Red Count minus Overlap Count = pure Red population).
 - ii. To calculate the total cell number, take the sum of Red plus Green counts and subtract the Overlap count. This will correct for the cells that are counted twice because they are transitioning between G1/S and expressing both Red and Green.
- iii. Determine the Percent Total of Red/Green/Yellow counts by dividing the pure count by the total cell number. For example
$$\% \text{ Total Red cells} = 100 * (\text{Red Count} - \text{Overlap Count}) \div [(\text{Red Count} + \text{Green Count}) - \text{Overlap Count}]$$
- iv. Cell cycle populations can be determined using normalized counts.
 1. % Red Object Count–cells in G1 phase
 2. % Green Object Count–cells in S/G2/M phase
 3. % Overlap Object Count–G1/S phase
- c. Note, basic analyzer method will not account for "colorless" (M/G1 and non-transfected) cells.

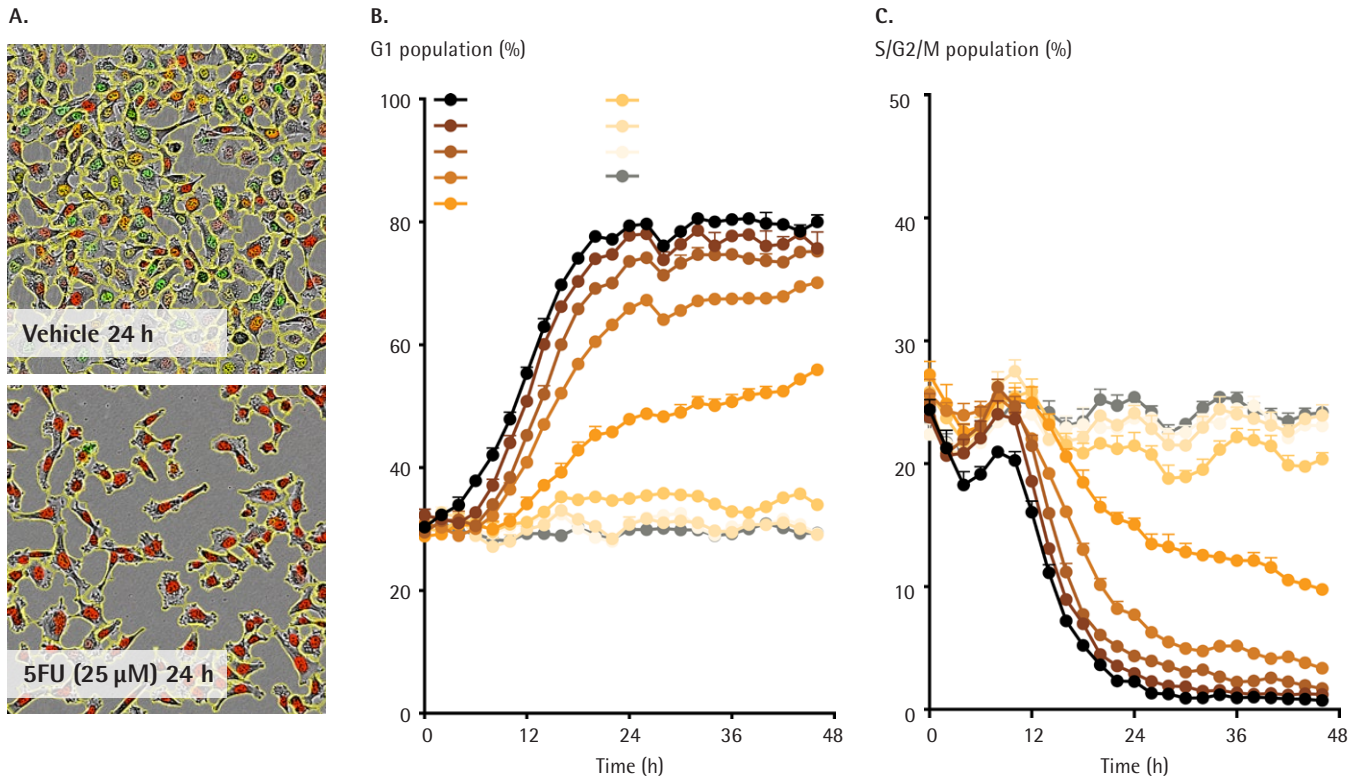


Figure 1: Effect of fluorouracil treatment on cell cycle. HT1080 cells transfected with the IncuCyte® Cell Cycle Red/Green Lentivirus Reagent were treated with increasing concentrations of fluorouracil (5FU), and phase, red and green fluorescence images were collected on the IncuCyte® Live-Cell Analysis System. Images were analysed using adherent Cell-by-Cell Analysis for individual cell masking (A), where cells were classified based on red and green fluorescence intensity. Time-course graphs (B&C) show a concentration related effect on the percentage of cells in the G1 (red) and S/G2/M (green) phases. Data shown as mean ± SEM of 3 wells.

A complete suite of cell health applications is available to fit your experimental needs. Find more information at www.sartorius.com/incucyte

For additional product or technical information, please e-mail us at AskAScientist@sartorius.com visit our website at www.sartorius.com or call
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